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# AN EXAMINATION OF THE FROG HEART ASSAY AND ITS POTENTIAL TO DETECT CARDIOACTIVITY

BY

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THESIS

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to mom and dad for making it possible
and
to lori for making it fun

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## INTRODUCTION

The idea that medicinal products may be found from the extracts of marine animals is not new.[1,2] Thus, it is worth spending considerable time and effort on developing accurate assays to test relatively pure compounds that are suspected of having biological activity. Before this, however, crude extracts must be screened to give at least a qualitative measure of the amount of activity a sample may possess. Also, so that the assay may be used in the field to give the researcher an idea of which organisms to collect, the assay must be relatively easy to perform, relatively fast, and the equipment must be compact. One such assay is the frog heart assay. The potential of detecting cardioactivity of marine natural products has been realized in recent years through the discovery of toxins of the sea anemones Anemonia sulcata, Anthopleura xanthogrammica, and Anthopleura elegantissima,[3] all members of the Coelenterata phylum. The toxicity of such animals from either contact or ingestion has been known for many years, thus it is not surprising that they have captured the interest of pharmacologists.[4] Three different toxins, ATXI, ATXII, and ATXIII, have been isolated from Anemonia sulcata and exhibit varying degrees of biological activity.[5] Studies with mice have shown that the dosage lethal to 50% of the mice tested needs to be about 25 times greater for ATXI than ATXII. Post mortem examinations of these mice have shown contracture of the heart, indicating cardiotoxic action of these toxins.[4] Such tests indicate that ATXII is about 100-300 times as effective as ATXI on left atria of guinea pigs and rats.[3,4] Upon further investigation, ATXII was found to exhibit a positive inotropic effect without destroying the ATPase, making it superior to the cardiac glycosides.[3,4,5] Extracts of both Anthopleura xanthogrammica and

Anthopleura elegantissima have shown a strong positive inotropic effect yet no chronotropic effect. As with the ATX toxins, it was found that the sodium-potassium ATPase was not inhibited.[4].

The frog heart assay used in this experiment was initially developed by Prosser and Shain at the University of Illinois, and has been modified by Traeger and, again, by Catlow.[6,7] There are several advantages to using a frog's heart. First, the frog's heart will survive for hours if placed in a bath of well-oxygenated Krebs solution. This relieves the burden of having the heart constantly needing to be perfused in blood as with a mammal's heart.[8] Also, the price of a frog is relatively low, and they are easier to care for than mammals or most other laboratory animals. Theoretically, the assay should be a successful means of screening for possible cardiac drugs for humans as the frog's heart has been shown to react similarly to a human heart to certain cardiac drugs.[9] It has also been found that the metabolism of the frog's heart is similar to that of a mammalian heart.[8] This assay is not without limitations, however. For example, because the assay is not performed on the entire living animal, it is not known what other physiological effects the concentration necessary to elicit a cardiac response will have. On the other hand, because a whole heart is used as opposed to a piece of muscle, toxicity effects of samples are increased. Also, because the reversibility of binding of extracts to the frog's heart is not known, a new frog's heart must be used for each trial. This will tend to increase error and must be corrected by doing several trials of the same sample. Also, the metabolism of the frog's heart is sensitive to both days in captivity and temperature of the surroundings.[8] Although it is relatively easy to

control the temperature, it is more difficult to keep the length of captivity of each frog constant.

Previously, the results of this assay have not proven to be very promising. The main problem is that the assay was shown not to be reproducible within acceptable limits. Indeed, some extracts were shown to elicit a positive increase in heart rate in one trial and a negative response in another. The focus of this research endeavor was to make the frog heart assay reliable enough to screen crude extracts for cardioactivity in order that they could be purified and identified. It is instructive, however, to first have a knowledge of the general physiology of the heart and the modes of action of some classic neurotransmitters and drugs.

## BACKGROUND

### A. Physiology of the Heart

The heart is mostly made up of cardiocytes, which are the muscle cells of the heart. Tissues which make and propagate electrical activity , made of "pacemaker cells", compose the rest of the heart. Each cardiocyte is bounded by the sarcolemma, which is a bilayered phospholipid-containing structure. The sarcolemma is filled with bundles of myofibrils, which are the contractile units of the cardiocytes.[10] Myofibrils are composed of sarcomeres, which are the functional units of the heart (Figure 1). During contraction, the Z-lines move together while the I and H bands decrease in size. The A-band remains the same size throughout contraction. Troponin and tropomyosin are important regulatory proteins for contraction and will be discussed shortly.[11]

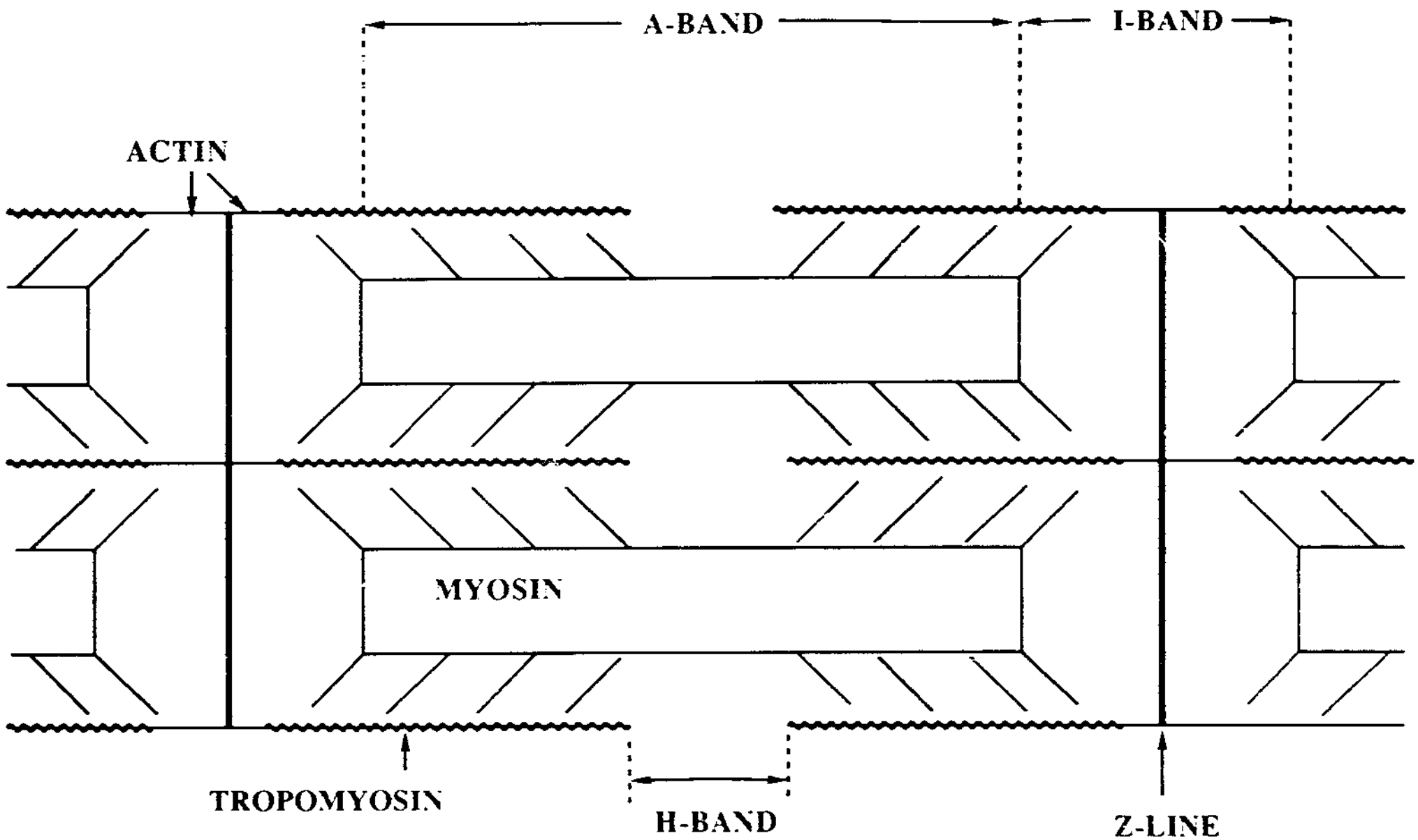



FIGURE : 1
THE STRUCTURE OF A SARCOMERE

The "pacemaker cells" make up the origin of the electrical activity of the heart. These cells are in the sinoatrial (S-A) node, which may spontaneously generate a beat, thus differentiating heart muscle from skeletal muscle.[10] This electrical activity goes quickly from the sinoatrial node to the atrioventricular (A-V) node and then through the bundle of His to the Purkinje fibers. The Purkinje cells, with their tight packing and intercalated discs, allow the heart to act as a synchronous unit.[10,11]

Once the current reaches the cardiocytes, the electrical activity is converted to mechanical activity via several ion pumps and channels located in the sarcolemma. One of the most studied of these pumps is the sodium-potassium pump or the sodium-potassium ATPase, as it utilizes ATP as its form of energy. The sodium-potassium ATPase is a globular protein which penetrates through the entire lipid bilayer of the sarcolemma. When bound by either a potassium ion or sodium ion, a conformational change occurs which then activates the ATPase.[10] The pump actively transfers three sodium ions to the outside of the cell for every two potassium ions pumped into the cardiocyte. This sets up an electrochemical gradient.[12]

A voltage-gated calcium ion channel, which is coupled to a sodium ion channel, is also present in the sarcolemma and is the main entrance for calcium ions into the cardiocytes. These calcium channels are opened due to a change in voltage across the membrane due to the opening of the sodium channel.[10] Although the amount of calcium ions entering the cell in this fashion is rather small relative to the amount of calcium in the cell, it is generally believed that it is the rate of change of calcium ion concentration which is vital in the contraction mechanism.[11,13]

The contraction of the heart is due primarily to the contractile proteins actin and myosin, and the regulatory proteins troponin and tropomyosin. Contraction depends on the ability of myosin to bind to actin. Tropomyosin is an alpha-helical protein wound around actin. Troponin is bound to the tropomyosin and has a site for binding calcium ions and a site inhibiting the binding of myosin to actin. When the concentration of calcium rises in the cytosol, the calcium ions bind to their respective sites on the troponin giving rise to a conformational change in both troponin and tropomyosin. This change in conformation uncovers the binding site for myosin on actin and contraction proceeds.[11,14]

The sarcoplasmic reticulum (SR) is an organelle of the cardiocyte which is very important in the contractile mechanism. Its main function is the regulation of the supply of calcium ions to the sarcomeres. During contraction (systole) the cytosolic calcium ion concentration (i.e. the concentration inside the cell yet outside the SR) is relatively high, and during relaxation (diastole) this concentration is relatively low.[15] This change in calcium ion concentration may be attributed to the SR as it is rich in calcium, yet its concentration of calcium ions has been shown to decrease before contraction.[11] The calcium pump of the SR is regulated by phospholamban, a phosphoprotein embedded in the membrane. Phospholamban, upon phosphorylation by ATP, will increase the activity of the pump. The mechanism governing this reaction is not well understood.[10] Also, it has been proposed that the calcium ions binding troponin come from the SR and that the calcium ions transported through the sarcolemma aid in the SR discharge. This mechanism is known as calcium-induced calcium release.[13]

The ability of membranes to increase spontaneously their permeability to sodium and potassium ions provides the basic mechanism of excitation of the heart. The cardiac cycle through systole and diastole shows the electrophysiological changes and is divided into five main phases. The resting potential is determined by the potassium ion gradient, which has a resting potential of -90 mV, as it is more permeable than sodium when the muscle is in its resting state. The combination of phases 1 and 2 make up the absolute refractory period during which the heart may not be stimulated. Phase 3 is also known as the relative refractory period during which a very strong stimulus may excite the tissue. After phase 3 there is a period of supernormal excitability such that a very weak stimulus may excite the heart.[16]

B. Pharmacology of the Heart

Pharmacology is generally based on a hormone-receptor theory. The models for such a mechanism are analogous to the enzymatic models known as the "lock-and-key" model and the "induced fit model". For the former, the agonist molecule (i.e. hormone or drug used to elicit the desired response) is considered as the key, while the receptor is thought of as the lock. The induced fit analog states that the agonist gives rise to a change in conformation in the receptor to cause an intracellular effect.[10]

Antagonist molecules are those molecules which interfere with the binding of the agonist and its receptor. This interference could be for the same site (competitive inhibition) or for a different site on the receptor.[10] Also, the degree to which the antagonist interferes with the agonist depends on their respective binding constants.[17]

Although these aforementioned models provide convenient mechanisms for the action of hormones and drugs, they are far from complete. For example, neither model is able to account for the almost "all-or-none" effect of a particular agonist. The allosteric theory, which postulates that the agonist changes the conformation of the receptor in such a way as to impede further agonist binding, attempts to correct for this. Otherwise, the maximum effect of an agonist would come from maximum occupancy of the receptor. The dose-response curve, by plotting the effect of an agonist versus concentration of that agonist, shows quite explicitly that a small change in concentration may lead to a large change in response.[10]

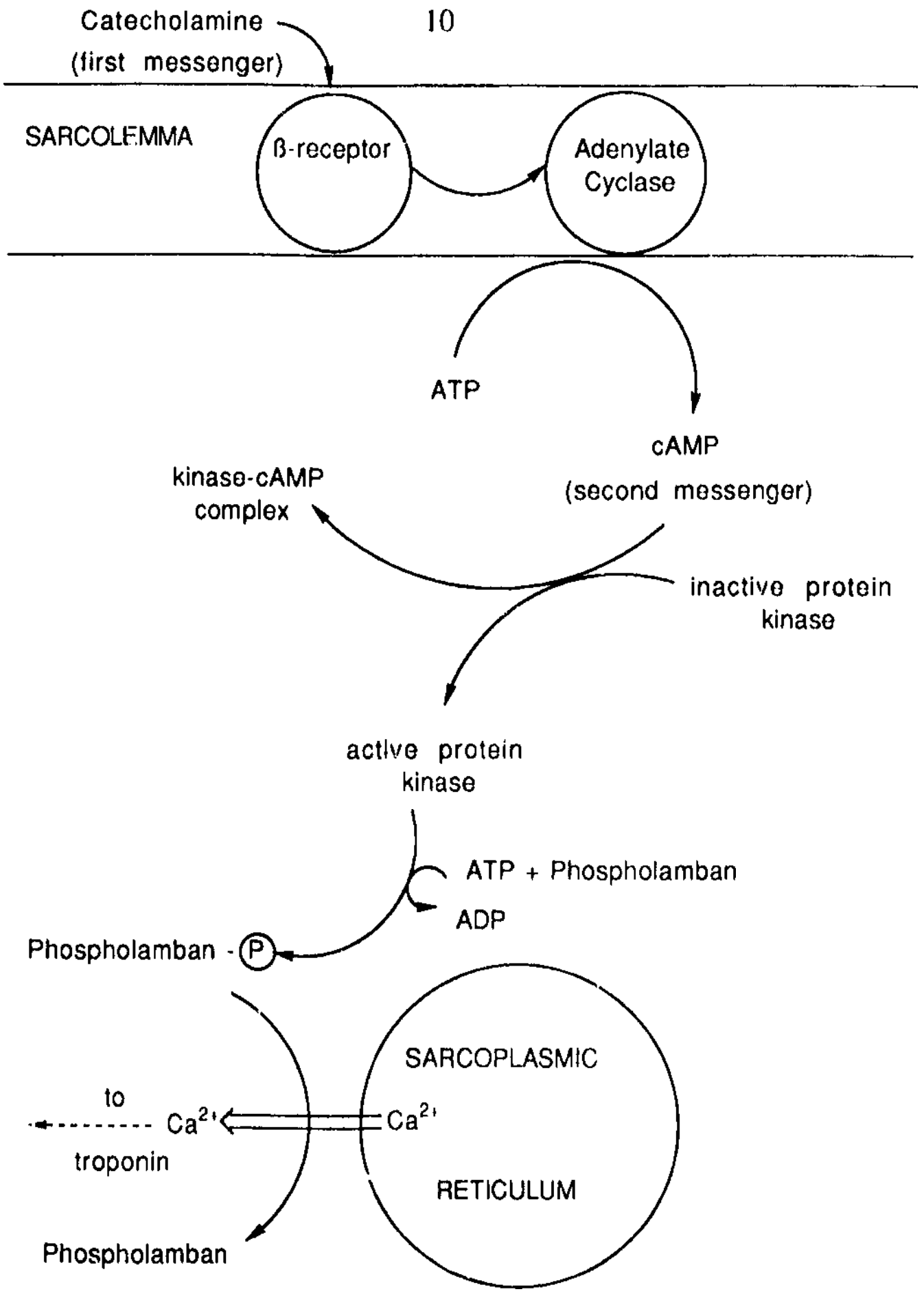
The mechanisms of the effects of hormones and drugs on the heart is readily explained by considering the second messenger concept. In this model, the signal of the agonist is called the first messenger, and the second messenger acts within the cell as it is formed at the membrane.The second messenger may produce cascade effects in that only a small amount is needed to elicit many different responses. As these responses give rise to other responses it is easy to see the great effect of just one messenger. This concept is used to explain the modes of action of both neurotransmitters and cardiac drugs.[10]

Only some effects of some catecholamines may be explained by the second messenger concept. For example, some catecholamines, upon stimulation of receptors of pacemaker cells as part of the sympathetic nervous system, increase the electrical impulse of the S-A node. This has the effect of increasing the heart rate as conduction of the A-V node is also enhanced.[18] This positive chronotropic effect is representative of the catecholamines, but is more of an indirect effect than that brought

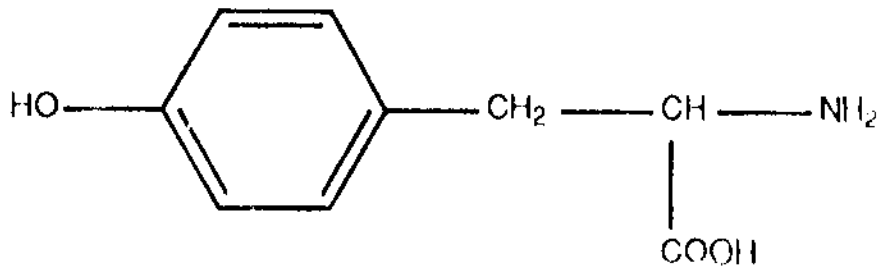
about by a second messenger. However, the positive inotropic effect (increased force of contraction), also representative of catecholamine stimulation, is readily explained by considering second messengers. In this case, the second messenger is cyclic adenosine monophosphate (cAMP), which is derived from ATP via the enzyme adenylate cyclase.[10] The stimulation of beta-adrenergic receptors by some catecholamines activates adenylate cyclase, thus producing cAMP. Inactive protein kinases are activated upon interaction with cAMP. The mechanism of the positive chronotropic effect is now easily imagined: the active protein kinases may aid in phosphorylating phospholamban to facilitate calcium release from the sarcoplasmic reticulum (Scheme 1).[19] The clinical uses of catecholamines include treatment for shock occurring from reduction of cardiac contractility, or A-V node conduction block.[16]

Norepinephrine, a catecholamine which is an extremely important neurotransmitter, is released from sympathetic nerve-end stores in emergency situations.[12] It is derived in the body from tyrosine; Scheme 2 shows the structural similarity of tyrosine, norepinephrine and epinephrine.[20] The half-life of norepinephrine is rather short, thus lending to its importance as a quick, short lived stimulator. Its effects on the body are seen only a few minutes after clinical administration.[16] The sympathetic division of the autonomous nervous system, due to the releasal from the adrenergic nerve-ends, is said to prepare the body for "flight or fight" .[12]

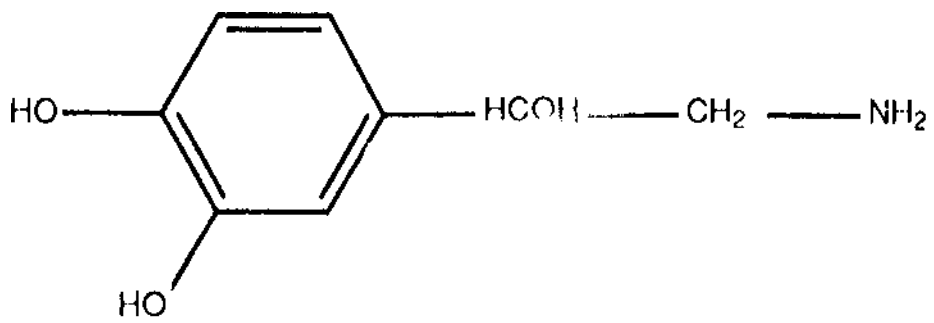
The other division of the autonomic nervous system, the parasympathetic division, is sometimes called the cholinergic nervous system. It is this system which gives rise to both negative chronotropic and inotropic responses, and a decrease in the conduction velocity of the



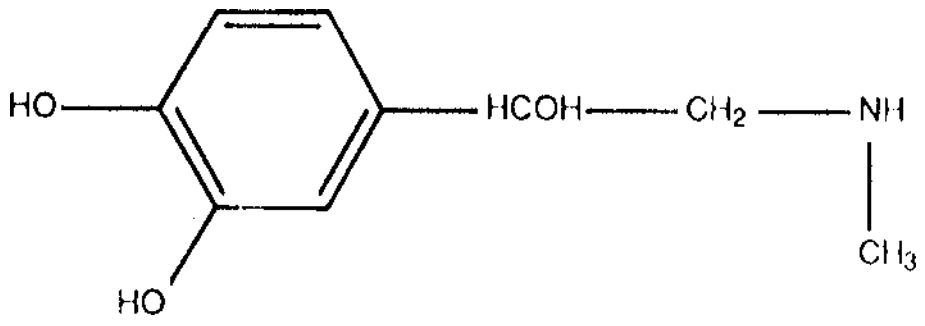

SCHEME 1 : POSSIBLE MECHANISM FOR POSITIVE INOTROPIC EFFECT OF CATECHOLAMINES




TYROSINE




NOREPINEPHRINE

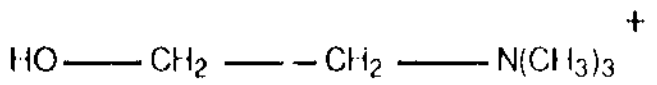



EPINEPHRINE

SCHEME 2 : STRUCTURES OF TYROSINE, NOREPINEPHRINE, AND EPINEPHRINE

A-V node.[10,18] The main neurotransmitter of this system is acetylcholine. Choline may be converted to acetylcholine in the presence of acetylcholine transferase and acetyl CoA (Scheme 3).[18] The cholinergic receptor of acetylcholine with respect to its effects on the heart is the muscarinic receptor, which acts on the vagus nerve. Stimulation of these nerve endings gives rise to the negative inotropic effect. It is also known that atropine is an effective blocking agent of the muscarinic receptor.[18] Acetylcholine is not readily administered as a cardiac drug, but its implications are important due to the cholinergic-like actions of some glycosides and anti-arrhythmic agents.[16,18]

The cardiac glycosides, or digitalis glycosides, were recognized to have medicinal effects before 1500 B.C..[16] The glycosides contain an aglycone moiety joined to a number of sugar molecules at the C3 position. Two typical glycosides, digoxin and digitoxin, are pictured in Scheme 4. The two main effects of digoxin therapy are a positive inotropic effect and a negative chronotropic effect.[18] The mechanism of action giving rise to the positive inotropic effect is rather controversial, though one model has been most widely accepted. It is known that there is a relatively high affinity, high specificity interaction between glycosides and the sodium-potassium ATPase inhibitory site.[21] Therefore, it has been proposed that glycosides act by inhibiting this pump and thus allowing a buildup of cytosolic sodium ions. To relieve this added stress, the sodium ions may leave by means of the sodium channel; but as this is coupled to the calcium channel, calcium ions will enter the cardiocyte.[16,18] As stated, this change in calcium concentration, albeit small, may cause the sarcoplasmic reticulum to release a relatively high concentration of calcium ions, thus increasing contraction (Scheme 5).

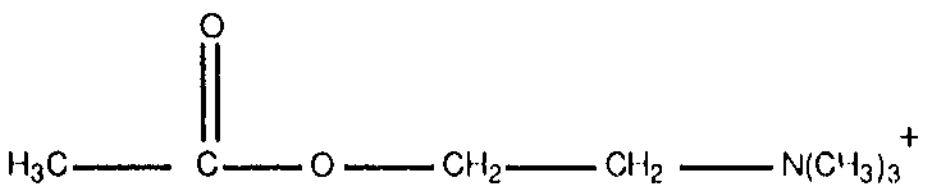


CHOLINE

+

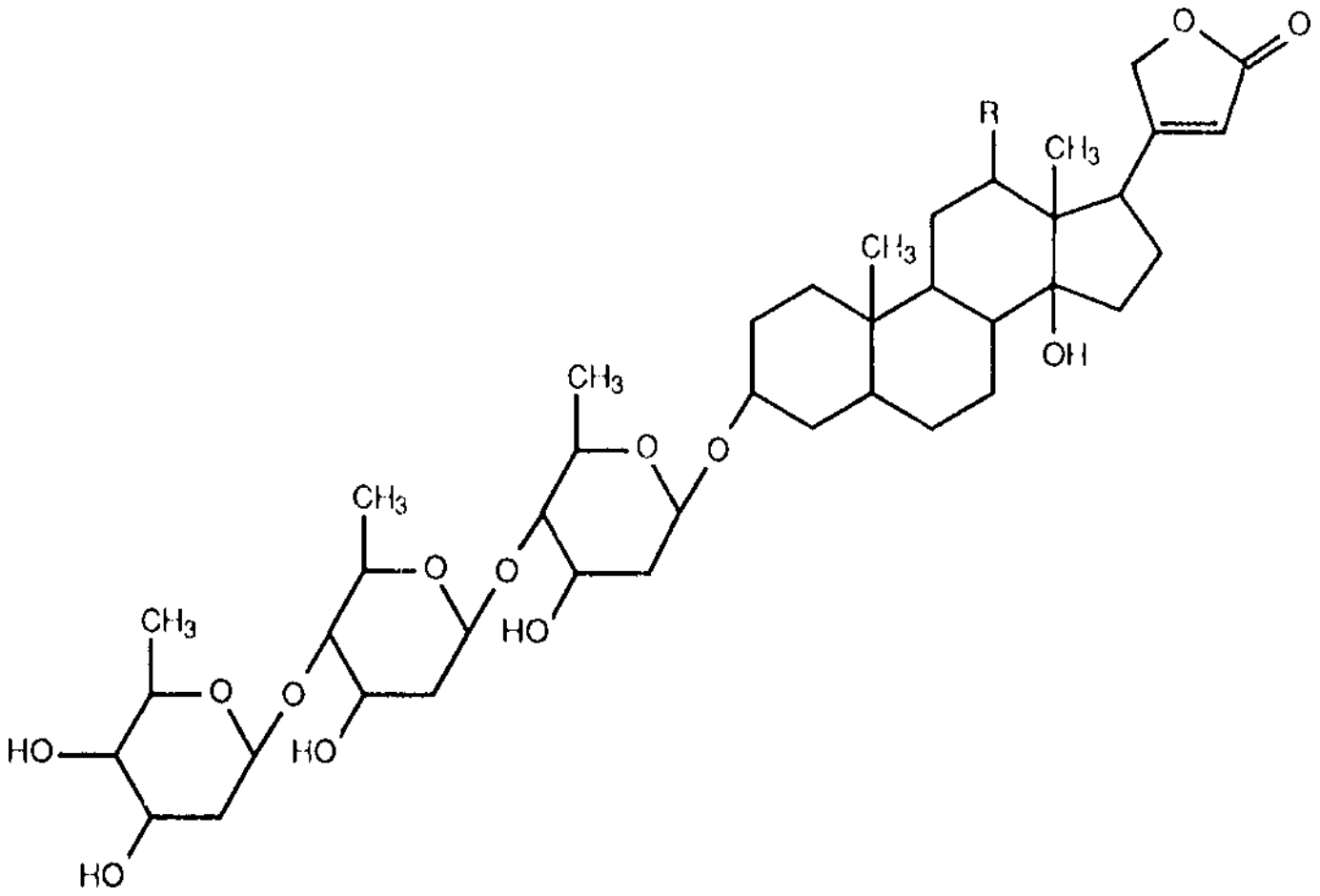
ACETYL CoA

CHOLINE ACETYL TRANSFERASE



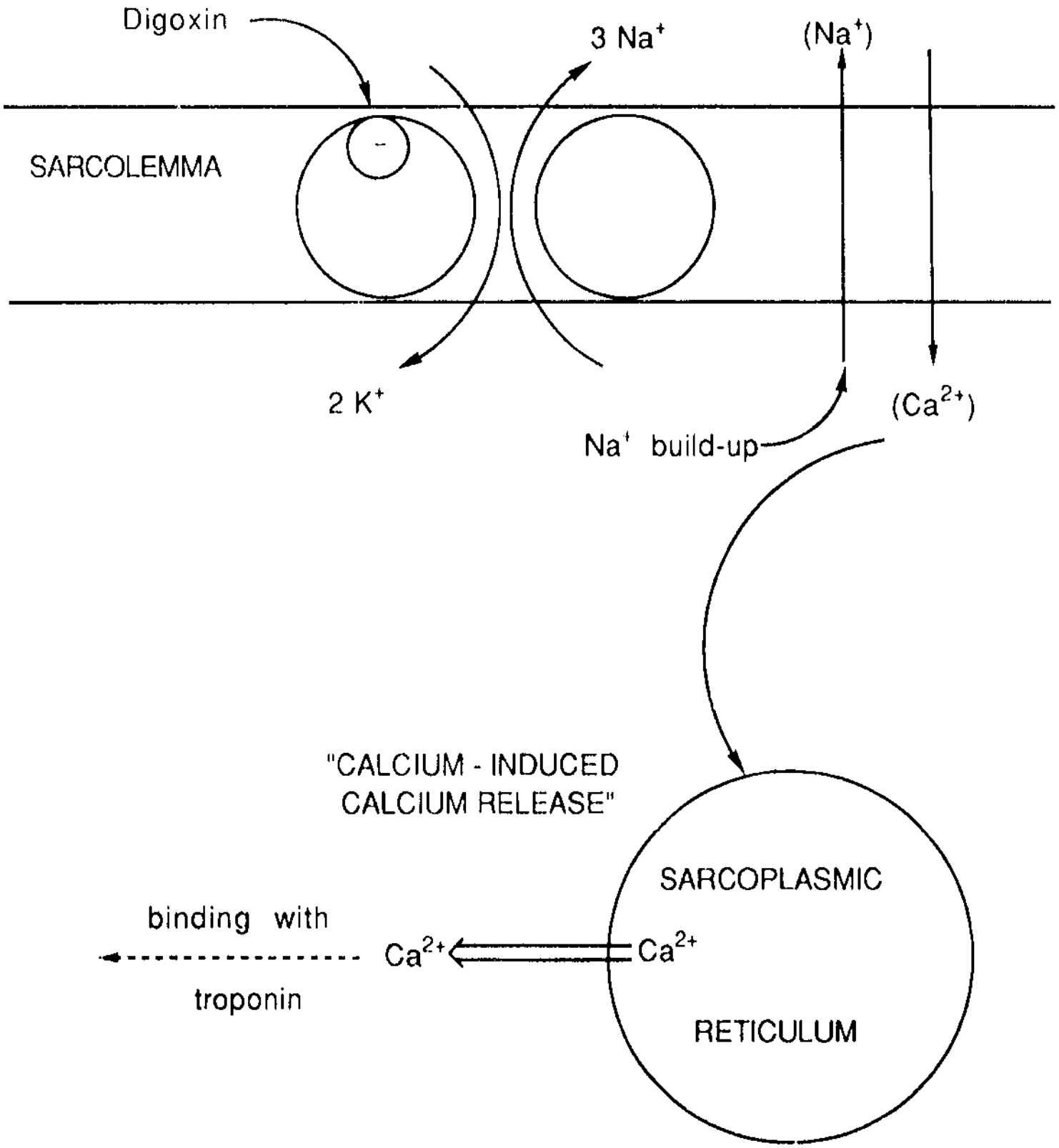
ACETYLCHOLINE

SCHEME 3 : CONVERSION OF CHOLINE TO ACETYLCHOLINE




DIGOXIN : R = OH
DIGITOXIN : R = H

SCHEME 4 : THE STRUCTURES OF DIGOXIN AND DIGITOXIN




SCHEME 5 : POSSIBLE MECHANISM FOR POSITIVE INOTROPIC EFFECT OF DIGOXIN

The negative chronotropic effect is said to come from decreased conduction through the A-V node. This is accomplished by the indirect vagal stimulation representative of the glycosides, as they allow the release of acetylcholine.[18] For this to be true, the increase in force of contraction from the glycosides must be greater than the response to acetylcholine as it generally causes a negative inotropic response.

A major problem with the clinical application of cardiac glycosides is that there is a fine line between therapeutic and toxic doses of these drugs.[21] As a matter of fact, it is generally believed that the therapeutic doses negatively affect the heart, though not to the extent of cardiac arrest.[18] This gives added interest in developing ATX as a cardiac medication. Obviously, then, if these types of drugs are to be considered, the benefits must surpass the ill effects. Digitalis compounds are quickly being replaced by drugs such as dopamine for the treatment of acute heart failure; but for chronic heart failure, it is still the drug of choice.[22]

Other drugs for the heart include anti-arrhythmic drugs, beta-adrenergic blocking agents, hypotensive drugs, vasodilators, calcium antagonists, and nitrates.[22,23] As these agents were not immediately relevant to the present research, a description of their mechanisms and uses will not be included.

## EXPERIMENTAL

The recording instrument first used by Catlow was a single channel minirecorder along with a graphic driving amplifier (GDA).[7] The GDA proved to be inadequate as it did not have the capacitance to properly filter the signals. This showed up as an exponential loss of power which could mistakenly lead one to believe that the force of contraction of the

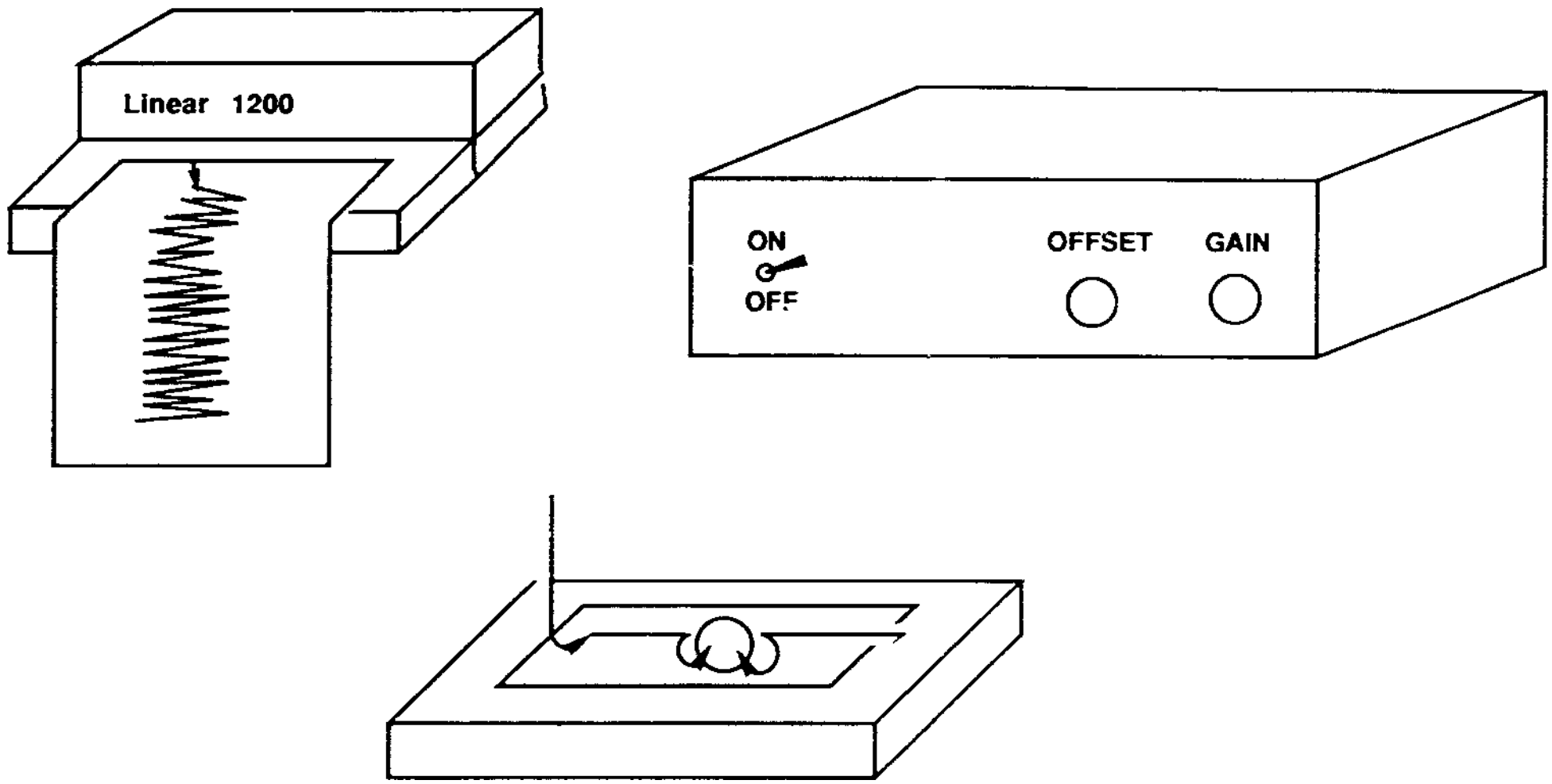
frog's heart was decreasing. A new amplifier was made by Reiner at the University of Illinois. A new linear 1200 recorder was used when the minirecorder began to malfunction. Although it was eventually repaired, it was no longer used as the 1200 is a superior recorder. The Grass FT .03 force transducer, which had been used in previous years, was used throughout this experiment.

The Plexiglass chamber was chosen for its compact size, lending itself well for work in the field. A small hole was drilled in one side to fit a hook on which to secure one end of the heart. Another hook was attached to the transducer to fix the other end of the heart. The experimental set-up is illustrated in Figure 2.

The chamber, or bath, was filled (30 mL) with Krebs solution, which consists of 89.5 mM NaCl, 3.0 mM KCl, 3.0 mM $NaHCO_3$, 3.0 mM D-glucose, 2.5 mM $CaCl_2$, and 1.0 mM $MgSO_4$ in distilled water. This solution was stored in a cold room (50 °F) when not in use to slow sugar degradation. Before an assay, the solution was sufficiently oxygenated with a 95:5 oxygen:carbon dioxide mixture.

The frog under study was the leopard frog, Rana pipiens pipiens. The size of the frogs usually varied between 3 and $3^1/_2$ inches although smaller frogs were sometimes used if they were all that was available. The frogs were kept in a cold room (50 °F) when not needed as this kept their metabolism low, thus making it much easier to care for them.

To perform an assay a frog would first be decapitated and its spinal cord pithed. The frog is then placed on its back, and both the skin and underlying layer of tissue were cut down the middle of the frog. Two horizontal cuts were made near the top of the thoracic cavity so that the heart was exposed. The pericardium, which is the transparent layer of




(NOT TO SCALE)

FIGURE 2 : THE FROG HEART ASSAY

tissue immediately surrounding the heart, was now carefully cut. Using forceps, the heart was taken by the carotid trunk and any attached tissue was cut. The heart was then placed in a container of well-oxygenated Krebs solution so that the heart could clear itself of blood. Once accomplished, the heart was pierced on both sides of the ventricle with tiny glass hooks with thread tied to them. The heart was now positioned in the bath so that the beating heart provided enough tension in the threads to show a strong response on the recorder, though not so much as to over stretch the tissue. The heart was now allowed to stabilize such that the change in frequency and amplitude over time was zero. This generally took fifteen to twenty minutes. At this time a sample was introduced to the bath. The crude extracts were prepared by grinding 2 g of a specimen in 20 mL of 3:1 : methanol:toluene solution. This solution was stored below 0 °C when not used as the crude extracts were of unknown composition, thus the degree to which any components of the extract were sensitive to heat was not known .

## RESULTS

The results of assays initially performed in order to reproduce previous data are given in Table 1. Data from assays testing the effects of norepinephrine bitartrate and digoxin are given in Tables 2 and 3, respectively. Also, dose-response curves were made for each of these drugs, as well as a graph showing the decomposition of norepinephrine over a one day period (Figures 3-5).

Results of assays testing sea urchins and sea anemones collected from Maine in 1986 are given in Table 4. Different fractions of the sea urchin 28-VI-86-1-5 were taken and tested. Results of these assays are

# TABLE 1
# INITIAL ASSAYS TESTING POTENTIALLY CARDIOACTIVE EXTRACTS (IBE 1983, INAE 1985 AND INAE 1986)

| SAMPLE # | % CHANGE IN FREQUENCY (AFTER 15 MIN.) | % CHANGE IN INTENSITY (AFTER 15 MIN.) | COMMENTS |
|---|---|---|---|
| | | | A. STEEL HOOKS |
| 28-VII-86-1-5 | -30.3 | -75.0 | sea urchin |
| 28-VII-86-1-5 | -30.0 | 0.0 | sea urchin |
| 3-VIII-86-1-9 | -58.5 | +16.7 | |
| 3-VIII-86-1-9 | -13.5 | +16.7 | |
| 3-VIII-86-1-6 | -33.0 | -66.0 | sea urchin |
| 29-VII-86-1-10 | -25.0 | +45.0 | |
| 12-VI-83-1-3A | -36.4 | -58.0 | Muricanthus princeps |
| 12-VI-83-1-3B | -68.6 | -72.0 | |
| 3-VIII-86-1-5 | -62.2 | -26.7 | |
| | | | B. GLASS HOOKS |
| 3-VIII-86-1-9 | -41.9 | -26.9 | |
| 3-VIII-86-1-9 | -54.3 | -42.4 | |
| 29-VII-86-1-10 | -45.7 | -88.9 | |
| 12-VI-83-1-3A | -35.5 | +4.0 | Muricanthus princeps |
| 12-VI-83-1-3B | -18.5 | -33.3 | |
| 28-VII-86-1-5 | -35.0 | -66.7 | sea urchin |
| 28-VII-86-1-5 | -27.0 | -20.0 | sea urchin |
| 3-VIII-86-1-5 | -21.9 | -8.3 | |
| 3-VIII-86-1-5 | -25.0 | -25.0 | |
| 6-VII-85-3-7 | -29.4 | -60.0 | Haliclona |
| 29-VII-86-1-10 | -30.0 | -10.0 | |
| 8-VI-83-1-3 | -50.0 | -57.6 | Aplidium |
| 8-VI-83-1-16 | -77.4 | -51.7 | Neorapana tuberculata |

# TABLE 2
# DATA USING NOREPINEPHRINE BITARTRATE

| CONC MOLARITY | MAXIMUM POSITIVE CHANGE(%) F | MAXIMUM POSITIVE CHANGE(%) I | OVERALL CHANGE(%) F | OVERALL CHANGE(%) I |
|---|---|---|---|---|
| $10^{-3}$ | 64.0 | 30.0 | 0.0 | -61.6 |
| $5x10^{-4}$ | 40.0 | 68.8 | 8.9 | 18.8 |
| " | 50.3 | 17.3 | 23.7 | -34.0 |
| " | 52.1 | -- | 21.6 | -- |
| " | 52.9 | 21.8 | 8.8 | -5.5 |
| " | 46.3 | 87.5 | 1.0 | -29.0 |
| " | 54.0 | -- | 17.0 | -- |
| " | 51.5 | 20.0 | 30.3 | -27.9 |
| " | 50.0 | 29.6 | 26.7 | -29.7 |
| "* | 35.1 | 24.0 | 21.0 | -28.2 |
| "* | 38.5 | 47.0 | -5.0 | -23.3 |
| "* | 30.8 | 21.8 | -12.3 | 0.0 |
| "* | 38.9 | 13.4 | -16.7 | -34.8 |
| "* | 31.6 | 63.4 | -7.9 | +23.2 |
| $10^{-5}$ | 36.0 | 11.8 | 12.5 | -19.4 |
| $10^{-7}$ | 10.4 | 10.4 | -17.3 | -1.0 |
| $10^{-8}$ | 7.5 | 10.4 | -20.0 | 22.4 |
| $10^{-9}$ | 3.8 | 0.0 | -26.9 | -6.3 |
| $5x10^{-4}$(avg.) | 49.6±4.52 | 40.8±29.8 | 27.3±9.54 | -17.9±20.6 |

* norepinephrine solution was one day old

-- : no data for that particular trial

change fifteen minutes after addition

# TABLE 3
# DATA USING DIGOXIN

| CONC MOLARITY | MAXIMUM POSITIVE CHANGE(%) F | MAXIMUM POSITIVE CHANGE(%) I | OVERALL CHANGE(%) F | OVERALL CHANGE(%) I |
|---|---|---|---|---|
| $10^{-3}$ | 16.7 | 0.0 | -46.7 | -75.6 |
| " | 12.2 | 0.0 | -52.7 | -72.0 |
| $10^{-4}$ | 5.6 | 0.0 | -41.7 | -42.7 |
| " | 3.8 | 0.0 | -38.8 | -94.0 |
| " | 6.3 | 0.0 | -34.4 | -40.2 |
| " | 10.2 | 0.0 | -30.0 | -29.5 |
| " | 6.3 | -- | -28.0 | -12.9 |
| " | 7.7 | 0.0 | -30.8 | -17.6 |
| $10^{-6}$ | 0.0 | 3.5 | -21.6 | -30.3 |
| " | 0.0 | 8.2 | -20.0 | 8.2 |
| " | 0.0 | 6.9 | -18.4 | -10.3 |
| " | 0.0 | 8.3 | -18.8 | -4.5 |
| " | 0.0 | -- | -25.7 | -14.3 |
| " | 0.0 | 8.6 | -21.4 | -8.5 |
| " | 0.0 | -- | -16.6 | -3.2 |
| $10^{-7}$ | 0.0 | -- | -13.5 | -- |
| $10^{-9}$ | 2.8 | 10.8 | -27.7 | 0.0 |
| $10^{-16}$ | 0.0 | 12.3 | -21.2 | -9.9 |
| $10^{-18}$ | 2.8 | 18.6 | -19.4 | -27.8 |
| $10^{-4}$(avg.) | 6.64±2.15 | 0.0 | 34.0±5.4 | -39.5±29.2 |
| $10^{-6}$(avg.) | 0.0 | 7.1±2.1 | -20.3±3.0 | -9.0±11.2 |

-- : no data for that particular trial
change fifteen minutes after addition

FIGURE 3 : DOSE-RESPONSE CURVE, NOREPINEPHRINE BITARTRATE

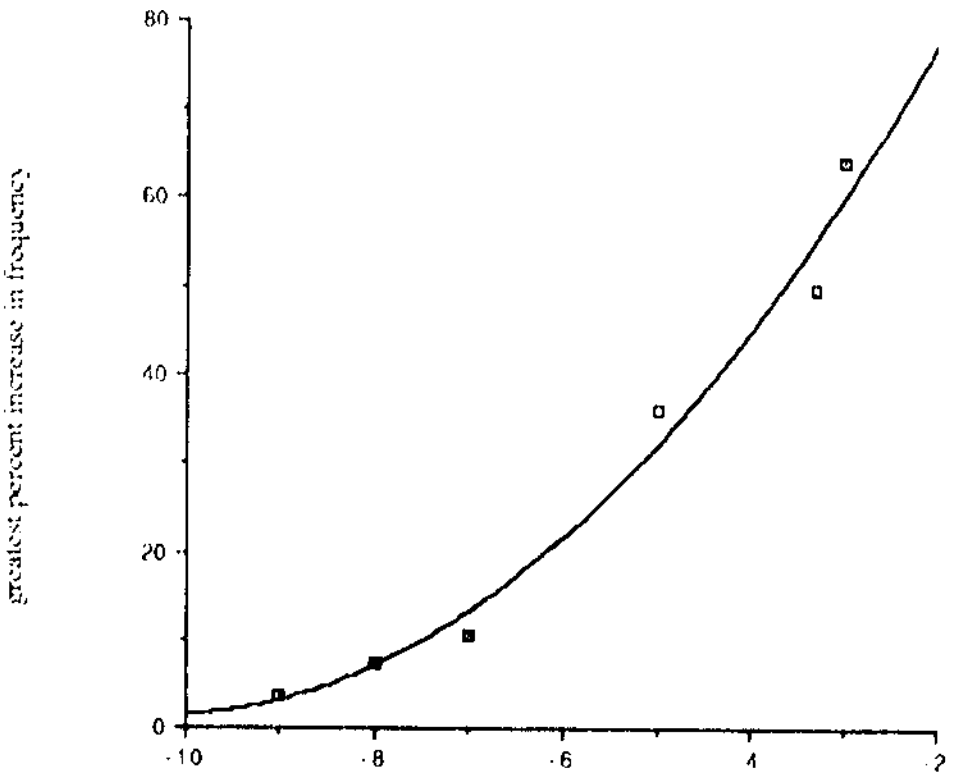



FIGURE 4 : DOSE-RESPONSE CURVE, DIGOXIN

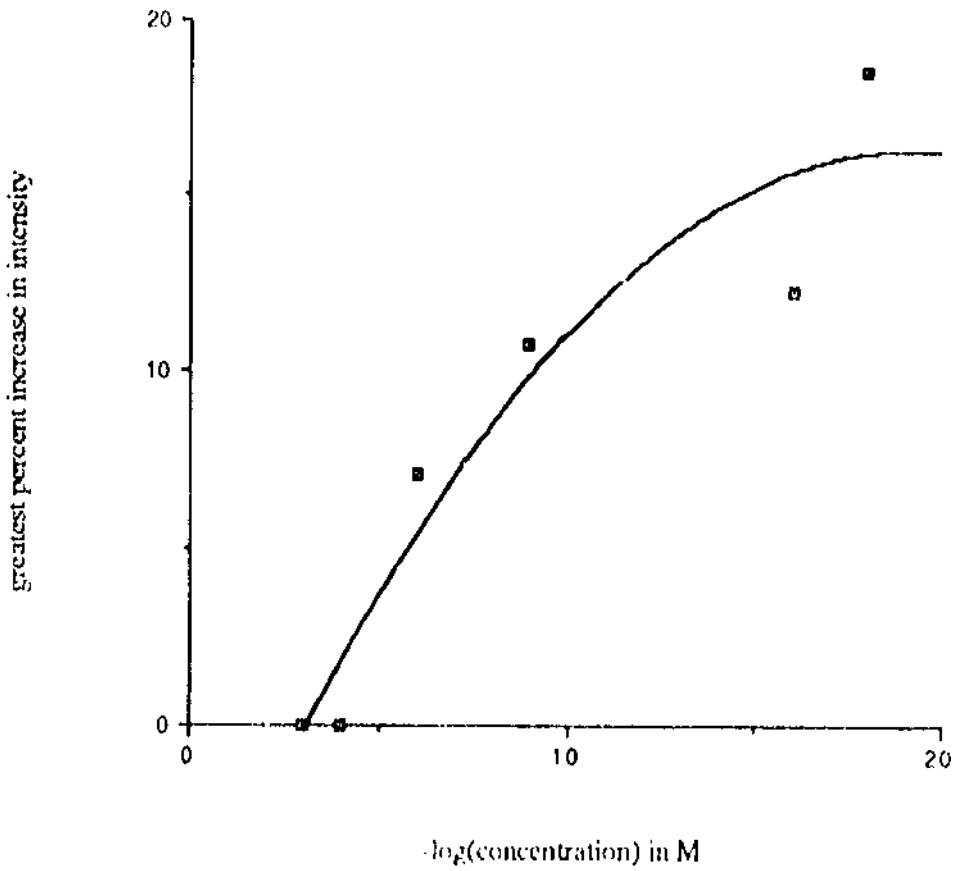


FIGURE 5 : NOREPINEPHRINE BITARTRATE (5x10 M)

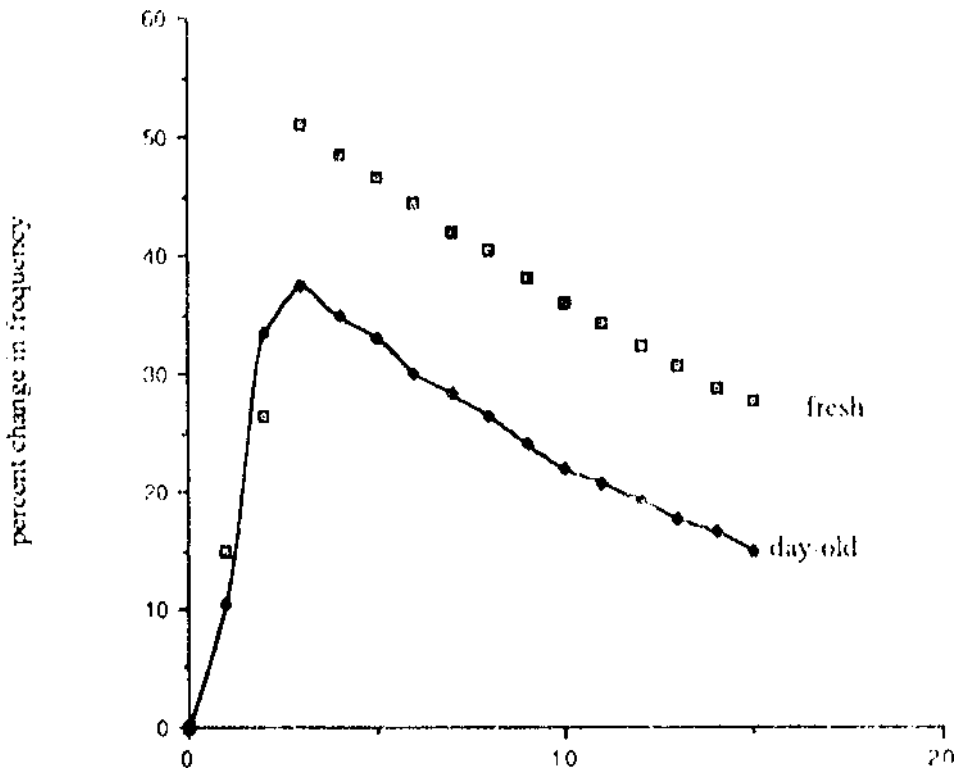
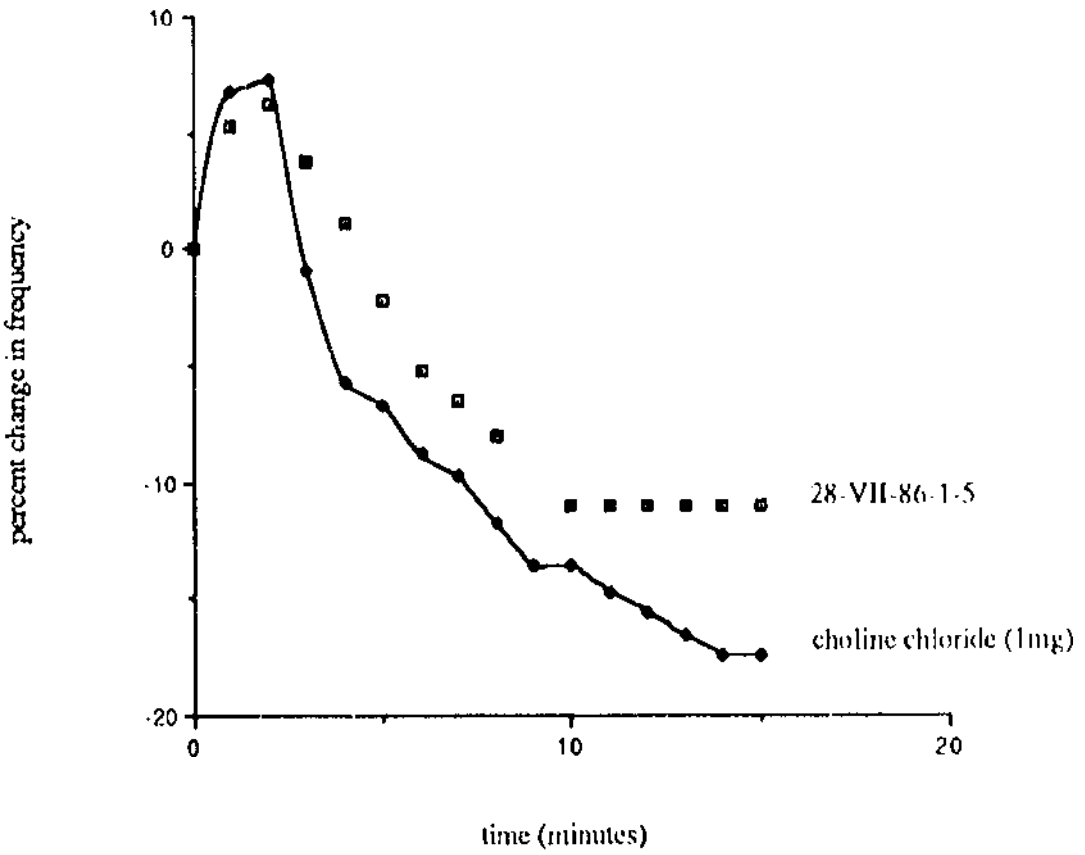



FIGURE 6 : CHOLINE CHLORIDE AND SEA URCHIN 28-VII-86-1-5



presented in Table 5. The extraction scheme is given in Scheme 6. Figure 6 shows the similar responses to choline chloride and 28-VI-86-1-5.

Data from assays performed using choline chloride, acetylcholine chloride, atropine, scopolamine, and/or 4-(1-naphthylvinyl)pyridine are given in Tables 6,7. Miscellaneous results from assays with 8,11,14-eicosatrienoic acid, the sea urchin 31-VII-86-3-14, a mixture of the three ATX toxins, tunicates from Florida, didemnins B and E, and various sponges are given in Table 8.

For the purposes of this paper, the term "intensity" is used in place of "amplitude" on all tables and figures. Thus, "I" is used in place of "A".

## DISCUSSION

The original goal of this endeavor was to re-test those extracts which had been shown to have cardioactivity in previous years. Once these results were shown to be reproducible, the rest of the project would consist of isolating and purifying the activity. The problem, however, was that although some samples had shown promise, the results were never sufficiently reproduced. Therefore, only the extracts which had shown a 50% change in either frequency or amplitude were to be tested. After testing several of these (Table 1), it was clear that there was too much error. Once the new amplifier was used, an inherent loss of frequency of the heartbeats was observed for 45 to 60 minutes after the frog's heart was placed in the bath and connected to the transducer. By counting the beats without inserting the steel hooks into the heart, it was noticed that the addition of DMSO did not cause the frequency loss. The idea that perhaps the heart muscle was stretched once set up was discarded as the frequency loss was noticed when the hooks were inserted but the heart

# TABLE 4
# DATA FROM SEA URCHINS AND SEA ANEMONES COLLECTED IN MAINE, 1986 (INAE 1986)

| ORGANISM | SAMPLE# | MAXIMUM SUDDEN CHANGE(%) F | I |
|---|---|---|---|
| SEA ANEMONE | 3-VIII-86-1-8 | 11.5 | 0.0 |
| SEA URCHIN | 28-VI-86-1-5 | 10.7 | 0.0 |
| SEA URCHIN (guts) | 31-VII-86-3-14 | 8.7 | 0.0 |
| SEA ANEMONE | 30-VII-86-2-3 | 8.1 | 0.0 |
| SEA URCHIN (spines) | 31-VII-86-3-14 | 7.0 | -17.2 |
| SEA ANEMONE | 29-VII-86-1-6 | 6.9 | 8.0 |
| SEA URCHIN | 2-VIII-86-1-1 | 6.3 | 6.2 |
| SEA URCHIN | 29-VIII-86-1-12 | 6.1 | 1.6 |
| SEA ANEMONE | 28-VII-86-1-7 | 6.1 | 0.0 |
| SEA URCHIN | 3-VIII-86-1-6 | 5.0 | 0.0 |
| SEA ANEMONE | 3-VIII-86-1-7 | 3.4 | 0.0 |
| SEA ANEMONE | 2-VIII-86-2-6 | 3.3 | 0.0 |
| SEA ANEMONE | 7-VIII-86-1-10 | 3.2 | 9.5 |
| SEA URCHIN | 7-VIII-86-3-5 | 3.2 | 0.0 |
| SEA ANEMONE | 7-VIII-86-3-3 | 2.5 | -- |
| SEA ANEMONE | 3-VIII-86-1-4 | 0.0 | 21.6 |
| SEA URCHIN | 2-VIII-86-2-5 | 0.0 | 0.0 |
| SEA URCHIN | 7-VIII-86-1-2 | 0.0 | -- |

-- : no data for that particular trial

each sample was approximately 3 mg

# TABLE 5
# DATA FROM VARIOUS FRACTIONS OF THE SEA URCHIN 28-VI-86-1-5

| FRACTIONS [a,b] | MAXIMUM POS. CHANGE(%) F | I |
|---|---|---|
| methylene chloride trituration | 8.3 | 0.0 |
| methanol trituration | 5.3 | 0.0 |
| precipitate | 0.0 | 13.3 |
| gonads | 14.3 | 0.0 |
| gonads | 8.8 | 0.0 |
| gonads | 9.1 | 0.0 |
| digestive tract | 0.0 | 0.0 |
| digestive tract | 3.3 | 0.0 |
| shell | 3.3 | 0.0 |
| shell | 0.0 | 0.0 |
| heptane layer of methanol:heptane mixture | 0.0 | --[c] |
| polar phase of extraction of methanol layer | 12.5 | 5.0 |
| " | 9.7 | 0.0 |
| " | 12.5 | 4.1 |
| non-polar phase of extraction of methanol layer | 0.0 | 0.0 |
| 100% water fraction through RP-18 | 6.9 | 0.0 |
| " | 4.9 | 0.0 |
| $H_2O$:methanol (70:30) through RP-18 | 6.9 | 0.0 |
| 100% methanol fraction through RP-18 | 8.3 | 1.7 |
| " | 5.6 | 0.0 |
| " | 10.5 | 0.0 |
| chloroform:methanol (1:1) through RP-18 | 0.0 | 0.0 |

[c] -- : no data for that particular trial
[a] each sample was approximately 2 mg
[b] see Scheme 6 for explanations

## TABLE 6
## DATA USING CHOLINE CHLORIDE AND/OR ATROPINE

| CONC. CHOLINE CHLORIDE | CONCENTRATION ATROPINE | MAX. POS. CHANGE PERCENT F | MAX. POS. CHANGE PERCENT I | OVERALL CHANGE PERCENT F | OVERALL CHANGE PERCENT I |
|---|---|---|---|---|---|
| $3.2\times10^{-3}$M | 0 | 19.0 | 0.0 | Died | Died |
| $3.2\times10^{-4}$M | 0 | 13.5 | 0.0 | -37.5 | -15.4 |
| $9.6\times10^{-4}$M | 0 | 10.6 | 0.0 | -26.3 | -- |
| $3.2\times10^{-5}$M | 0 | 8.3 | 1.7 | -16.7 | -18.8 |
| $3.2\times10^{-5}$M | 0 | 5.8 | 0.0 | -18.4 | -5.9 |
| $3.2\times10^{-5}$M | 0 | 13.7 | 0.0 | -17.2 | -15.0 |
| $3.2\times10^{-6}$M | 0 | 2.5 | 7.4 | -22.5 | -4.3 |
| 0 | $10^{-5}$M | 3.3 | 0.0 | 0.0 | 0.0 |
| 0 | $10^{-4}$M | 3.4 | 0.0 | -10.3 | -4.2 |
| 0 | $10^{-3}$M | 4.3 | -- | -8.7 | -- |
| $3.2\times10^{-5}$M | $5\times10^{-5}$M | 0.0 | 0.0 | -14.7 | -4.2 |
| $3.2\times10^{-5}$M | $10^{-4}$M | 0.0 | 0.0 | -12.1 | -8.9 |
| $3.2\times10^{-5}$M | $5\times10^{-6}$M | 3.3 | 0.0 | -13.2 | 0.0 |

change fifteen minutes after addition
-- : no data for that particular trial

## TABLE 7
## DATA WITH SCOPOLAMINE, ACETYLCHOLINE, AND 4-(1-NAPHTHYLVINYL) PYRIDINE

| CONC. MOLARITY | SAMPLE | MAXIMUM POS. CHANGE(%) F | MAXIMUM POS. CHANGE(%) I | OVERALL Δ (%) F | OVERALL Δ (%) I |
|---|---|---|---|---|---|
| $10^{-4}$ | Scopolamine | 9.4 | 0.0 | -9.4 | 0.0 |
| $10^{-5}$ | " | 6.7 | 21.6 | -13.3 | 21.6 |
| $1.8\times10^{-5}$ | Acetylcholine | Died | Died | Died | Died |
| $1.8\times10^{-8}$ | " | 11.9 | 11.5 | -5.6 | -9.2 |
| $10^{-4}$ | 4-(1-naphthylvinyl) pyridine | 0.0 | 15.0 | -30.0 | 15.0 |
| " | " | 3.8 | 0.0 | -35.0 | -- |
| " | " | 2.6 | 0.0 | -39.5 | 0.0 |
| $10^{-6}$ | " | 0.0 | 10.0 | -16.0 | -13.0 |
| $10^{-8}$ | " | 0.0 | 4.8 | -12.0 | -3.2 |

# TABLE 8
# MISCELLANEOUS DATA

| SAMPLE | COMMENTS | MAX. SUDDEN CHANGE PERCENT | | OVERALL CHANGE PERCENT | |
|---|---|---|---|---|---|
| | | F | I | F | I |
| 8,11,14 - eico-satrienoic acid | methyl ester | 0.0 | 0.0 | -16.0 | -5.0 |
| 31-VII-86-3-14 | Gonads | 12.1 | 0.0 | -6.1 | -34.7 |
| " | " | 10.5 | 5.3 | -5.3 | -2.3 |
| " | " | 6.3 | 0.0 | -12.5 | 0.0 |
| " | MeOH Phase | 6.5 | 9.2 | -12.9 | 0.0 |
| " | Heptane Phase | 0.0 | 3.9 | -9.5 | -9.2 |
| ATX mixture | 50 µg | 0.0 | 7.7 | -18.9 | -17.9 |
| 18-2-5 | Florida Tunicate | 3.1 | 4.8 | -12.5 | -2.9 |
| 17-1-10 | " | 2.9 | 7.2 | -17.6 | 3.6 |
| 17-1-5 | " | 6.3 | 0.0 | -18.8 | -15.0 |
| 17-1-6 | " | 2.3 | 8.0 | -11.6 | 1.6 |
| 17-1-4 | " | 0.0 | 2.6 | -14.3 | -10.5 |
| 17-1-7 | " | 6.5 | 6.8 | -19.4 | 2.9 |
| 17-1-3 | " | 0.0 | 10.0 | -21.6 | 10.0 |
| 18-2-2 | " | Died | Died | Died | Died |
| didemnin B | 10 µg | 0.0 | 0.0 | -2.4 | -8.3 |
| " | 50 µg | 0.0 | 2.0 | -14.6 | -16.1 |
| didemnin E | 10 µg | 0.0 | 3.0 | -5.3 | 0.0 |
| " | 50 µg | 6.7 | 0.0 | -3.3 | -12.5 |
| 29-VI-82-3-1 | Agelas (IBSBE) | 0.0 | 11.5 | -22.0 | 10.0 |
| 26-VI-81-1-4 | Neofibularia (IREC) | 2.9 | 4.7 | -17.4 | 0.0 |
| 29-VI-82-2-1 | Tedania (IBSBE) | -21.0 | 0.0 | -47.0 | -10.0 |
| 6-VII-81-1-2 | Tedania (IRCE) | 3.9 | -91.6 | -20.8 | -90.0 |
| AHCE 650 | Ptilocolis | 11.4 | -37.4 | 0.0 | -15.0 |
| AHCE 583 | Aplysina | 2.4 | 0.0 | -21.4 | -30.0 |
| AHCE 628 | Plakovtis | 0.0 | 2.6 | -37.5 | -16.9 |

change fifteen minutes after addition

was not connected to the transducer. Because glass has been shown to be less harsh on tissue than steel,[24] small glass hooks were made. Using these, the heart exhibited no loss of frequency nor amplitude after fifteen to twenty minutes. Some extracts were tested using the glass hooks, and the results were sometimes quite different than those using steel hooks (Table 1). Something that was apparent in almost all cases, however, was that there did not seem to be many relatively large changes in either frequency or amplitude, but the tests showed quite clearly a rather slow loss or gain during the fifteen minutes in which the heart beats were recorded. Also, there were very few instances in which the amplitude actually increased for the fifteen minutes of each trial, and no such instances for the frequency. By only taking the frequency and amplitude levels at five, ten, and fifteen minutes, as previously cited,[6,7] some potentially important data was lost because some samples seemed to show fairly sudden changes in the first few minutes. It is worthwhile, then, to record data at one minute intervals. Results of doing this and calculating percent changes gives some surprising results as will be discussed. One possible explanation for the apparent slow drifts toward negative changes is a change in pH of the bath,[24] but this was not thoroughly investigated.

It was decided that before attempting to test many different extracts and trying to draw meaningful conclusions, the assay must be tested to at least qualitate its reproducibility. Norepinephrine and digoxin were tested so that both increases and decreases could be observed. Because norepinephrine is insoluble in water, norepinephrine bitartrate was used, but will be referred in the text simply as norepinephrine. Upon testing norepinephrine, the most dramatic result was that both frequency and

amplitude decreased to some extent after the first initial increase (Table 2). In fact, at concentrations lower than $10^{-5}$M, both frequency and amplitude were at levels below their initial values. Addition of norepinephrine to water seemed to decrease the pH of the solution, but as the initial response was most important, this was not tested further. The results of change in frequency seem very reproducible (within 10%) for the first few minutes. The dose-response curve for norepinephrine indeed shows that the frequency increases with increasing concentration. Similar qualitative results with the amplitude are also found, but to a lesser degree. The reason for this is most likely attributed to the disturbance caused by the addition of the sample and subsequent stirring of the bath. At the time of addition, the heart is sometimes moved thus changing the tension on the threads. The apparent amplitude would then be different from its actual value. If the heart now moves in the bath, either to its original position or to a different one, an apparent change in amplitude may not be due to the heart beating, but to its moving in the bath. Using the present procedure of adding the extract , giving the exact same disturbance to the bath is not realistic. Correcting this problem would undoubtedly make the operation more complicated, which is not advisable for an assay which is to be used in the field. The problem, however, is mostly quantitative as it is seen that norepinephrine definitely gives rise to a positive inotropic response. Therefore, this assay does seem to give qualitatively expected results using a pure drug. It does not appear unreasonable to obtain results at least relatively quantitative, however. For example, norepinephrine readily decomposes; some trials were performed on a one day old solution of norepinephrine,

and the results relative to those with a fresh solution show the assay can detect differences in concentration (Figure 5).

Next, digoxin was tested. The results at first appeared less promising than those with norepinephrine. For example, at $10^{-3}$M and $10^{-4}$M, there was no increase in amplitude, yet an increase in frequency of the beats within the first few minutes. Because these are relatively high concentrations, and digoxin acts by destroying the ATPase, the amplitude data are not unreasonable as these concentrations are no doubt toxic to the heart. The increase in frequency may also be explained. At toxic levels of digoxin, the resting potential is decreased and the depolarization slope is increased.[18] Although never explicitly stated, it would seem that the heart would be able to beat faster as the ion equilibrium potential would not be such a burden. Since this is a toxic concentration, the increase should be temporary, and it in fact is. As the concentration is lowered to $10^{-18}$M, the amplitude increase is observed, and the change in frequency is not so dramatic (Table 3). The dose-response curve shows this assay is relatively sensitive to small concentrations of a pure drug.

Using both norepinephrine and digoxin, a few significant observations were noted. First, the initial frequency of the frog's heart is rather important with respect to its response. The usual initial frequency is between 35 to 40 beats per minute. When this frequency was below 25 beats per minute, the drugs seemed to affect the heart to a greater degree. Also, a battery had been used in previous years to try to re-start a heart which had undergone arrest. This usually gave inconsistent, even erratic, results. Hearts which had stopped were revived only by applying pressure at regular intervals, and even then this was done sparingly. Another observation was that by circulating the bath with a stream of oxygen as

had been done in the past, no results were obtained as this affected the transducer. This may cause a problem as the effective concentration around the heart may decrease over time; this could explain why values slowly decrease. If this is the case, it is not a very significant problem as long as this assay is able to detect a cardioactive agent and respond to it. Because of this, only the results within the first few minutes are deemed very significant. This is especially reasonable when dealing with toxic substances as each heart, once poisoned, may slow down or stop at different rates; and an examination of this is not the purpose of this assay.

Once the assay was trustworthy with pure drugs, it was ready to be tested with crude extracts. The first to be tested were samples of sea urchins and sea anemones collected in Maine in 1986 (Table 4). The results of these tests were quite startling. For example, 16 of the 18 samples showed some type of sudden change. Also noticed was that both the frequency and amplitude of the heart beats slowly decreased over time after the initial change. The sea urchin 28-VI-86-1-5 was tested as it showed the greatest response of the sea urchins. Sea anemones have shown cardioactivity in the past, thus a finding may not be novel.[25] First of all, the activity was shown to be reproducible. Next, the sample was triturated first with methylene chloride, and then with methanol. This left a precipitate. All of these were tested, and the activity was found in both solvents, though not in the precipitate. A frozen sea urchin was then thawed, and separated into shell, digestive tract, and gonads. These were ground and placed in a 3:1 : methanol:toluene solution (1 g extract to 10 mL solution). Upon assaying these, the activity seemed to be located in the gonads, and this result was reproducible. Next, using the extraction

scheme shown in Scheme 8,[26] the active component appeared to be rather polar. This scheme did not, however, effectively isolate the activity. Indeed, it seemed to be distributed in most fractions through the column. At this time, bioactive lipids from the gonads of the sea urchin Strongylocentrotus droebachiensis collected in Maine were reported.[27] These lipids are the cholesterol and triglyceride esters of 8,11,14-eicosatrienoic acid. The methyl ester of this acid was tested, but no activity was found. Data from mass spectra of the active portions of 28-VI-86-1-5 showed the possibility of cholesterol and choline. High resolution FABMS concluded that choline is indeed present.

Because the heart is able to convert choline to acetylcholine, this result is quite significant. Assays performed on choline chloride gave results similar to those with the sea urchin under scrutiny (Figure 6). Acetylcholine was also tested and found to be quite active even at small concentrations. Although acetylcholine should give rise to negative inotropic and negative chronotropic responses, these results may be explained by a rationale similar to that explaining the effects of digoxin. A toxic level of acetylcholine will most likely have initial unexpected results on the heart. Since digitalis compounds use the release of acetylcholine to exhibit their negative chronotropic effects, this is not an unreasonable line of thought. In order to try to block the muscarinic receptors, atropine was used. Unfortunately, reportedly effective levels seemed to show some degree of toxicity. Testing atropine and choline chloride, however, did seem to abolish the initial response, although the toxicity was still apparent (Table 6). The frog's heart, however, is known to destroy atropine,[8] thus, scopolamine was tested as it is not broken down by the heart.[24] Even small levels of scopolamine appeared too toxic

for the heart, however. Thus, the hypothesis of choline indirectly causing the effects could not be corroborated in this manner. Next, 4-(1-naphthylvinyl)pyridine was tested as it is reported to destroy choline acetyltransferase at concentrations at around $10^{-5}$M.[28] Upon testing as low as $10^{-8}$M, this compound appeared at least as toxic as the choline chloride. This is not too surprising as the reported assays were done on homogenated rat brains, thus the toxicity the compound may have solely on the heart would not be observed.

Some very important conclusions may be gathered from these experiments. For example, if the heart allows choline to be converted to acetylcholine, the observation of the slow negative drift of both frequency and amplitude may be explained. Choline is found in all animals,[24] which would explain the large number of samples found to exhibit some activity. This does, however, raise some doubt on the effectiveness of the frog heart assay.

As a test to see if the activity noticed in most of the sea urchins was similar, the sea urchin 31-VII-86-3-14 was subjected to an extraction similar to that of 28-VI-86-1-5, although the sample was not put through a column. These results show quite clearly that the activity stays almost exclusively in the polar phase, as seen with the other sea urchin. A mixture of the ATX toxins was also tested. Unfortunately, the exact proportions of each toxin is not known. Also, only 50 µg were available for the assay. Under the assumption that the sample consisted entirely of ATXII, the concentration would be the minimum required to show a response . Because ATXI is much less potent, and ATXIII does not show any activity,[3,4,5] it is not surprising that no dramatic result was found. Also tested were some tunicates collected from Florida in 1987-88. Some

showed results similar to those for the sea urchins and sea anemones, but as a whole seemed to be less active. Didemnins B and E also showed little, if any, activity. Finally, some sponges were tested with only one noticeable result : Tedania ignus (29-VI-82-2-1) gave rise to a 21% loss in frequency after only one minute.

Obviously, a major complication in an experiment dealing with live animals is the animals themselves. A few unexpected incidents served to slow down the project. For example, as cited, the metabolism of the frog's heart is somewhat dependent on days in captivity. It soon became readily apparent that the frogs must be kept for at least one to two days before attempting to use their hearts for the assay. Otherwise, the initial frequencies were too low to be useful. Also, the beginning of the winter season is an extremely bad time to have to depend on using the frogs. One reason is that they seem to be more susceptible to contract "red leg', a rather common disease due to contaminated water. It is advisable to put some copper wire in the tank with the frogs and to make sure there is a constant supply of running water.[24] Also, frogs are able to sense the seasons, and, unlike mice, cannot be conditioned in the laboratory setting.[24] Thus, during late autumn, they undergo hormonal changes because they are trying to hibernate and they exhibit a great deal of hyperactivity at this time. This condition usually lasts at least a couple of weeks.

## SUMMARY OF RESULTS

The most striking result from this research is that choline appears to give rise to a response in the heart, either directly, or indirectly, through acetylcholine. Although only one sample was definitely shown to indeed

contain choline, its universal presence in animals has been known for quite some time.[24] Also, the results of most all assays performed on marine samples qualitatively parallel the data found when choline chloride was tested. Though no conclusive evidence that choline was the cause of noticed effects was obtained, the hypothesis was never disproven either.

That choline may elicit an effect on this assay raises some question as to the value of this as an experimental tool. However, results from purified compunds seem to suggest that under certain conditions this assay is not only reproducible, but reacts to somes drugs as would be expected of a human heart. Although the results suggests that frequency data is more trustworthy than amplitude data, both are qualitatively reliable.

## CONCLUSIONS

The frog heart assay appears to be an excellent method of detecting cardioactivity in pure samples. This has been observed with nor-epinephrine, digoxin, and acetylcholine. Unfortunately, its potential as a field assay to screen crude extracts is very doubtful. One reason for this is that the entire heart is used, thus it is difficult to distinguish between actual cardioactive effects and simple toxicity to the heart. For example, since the heart depends on its ion channels and pumps, varying the concentration of certain salts will elicit a response, but there is no real clinical application of this. Also, if choline is indeed converted to acetylcholine in this assay, the sample tested must be extremely potent or at a high concentration to overcome this. Effects of compounds on the heart are also concentration dependent, and it is unrealistic to quantitate

amounts of sample in the field. Therefore, it seems that this assay would lend itself well as an initial test on somewhat purified unknown compounds.

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